UNIVERSIDADE ESTADUAL DE CAMPINAS

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OKEKE CHINEDU

POLYMORPHISMS IN HEME OXYGENASE-1 AND BONE MORPHOGENETIC PROTEIN RECEPTOR 1 GENES AND ESTIMATED GLOMERULAR FILTRATION RATE IN BRAZILIAN SICKLE CELL ANEMIA PATIENTS

POLIMORFISMOS NOS GENES DA HEMEOXIGENASE-1 E DO RECEPTOR DE PROTEÍNA MORFOGENÉTICA ÓSSEA 1 E ESTIMATIVA DA TAXA DE FILTRAÇÃO GLOMERULAR EM PACIENTES BRASILEIROS COM ANEMIA FALCIFORME

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DEDICATION

This Project is dedicated to Almighty God who strengthen me and in whom I trust.

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RESUMO

Estresse oxidativo causado por hemólise intravascular compõe a fisiopatologia das complicações renais na Anemia Falciforme (AF). Mutações que afetam genes envolvidos nas vias oxidativa e de sinalização podem estar associadas à doença renal nesta importante e frequente condição. Nós determinamos as frequências alélicas e genotípicas de alguns polimorfismos comuns encontrados nas regiões promotoras dos genes da Heme Oxigenase 1 (*HMOX1)* [SNP rs2071746 (A>T) e repetições (GT)n, alelos curtos e longos] e do Receptor de Proteína Morfogenética Óssea - tipo 1B (*BMPR1B*) [SNPs rs17022863 (A>G), rs4331783 (A>G) e rs1470409 (A>G)] em 75 pacientes AF adultos, em estado estável da doença e sem tratamento com hidroxiureia, e as comparamos àquelas encontradas em 160 controles saudáveis (doadores de sangue) provenientes da mesma região geográfica do país e com as mesmas características étnicas dos pacientes. Nós também investigamos se esses polimorfismos poderiam influenciar a taxa de filtração glomerular estimada (eTFG) para esses pacientes. Os quatro SNPs foram genotipados usando ensaios TaqMan, enquanto as repetições de *HMOX1* (GT)n foram determinadas por análise de tamanho de fragmento de PCR. A eTFG foi calculada pela fórmula de Modificação da Dieta na Doença Renal (MDDR). Todas as frequências genotípicas aqui determinadas encontravam-se em equilíbrio de Hardy-Weinberg. Em relação ao *HMOX1* rs2071746, verificamos que a mediana da eTFG foi significativamente maior nos pacientes com o genótipo TT (p=0,019), inclusive quando comparada àquela resultante da soma dos valores de eTFG dos pacientes com os genótipos AT e AA (p=0,009); em relação às repetições (GT)n, as medianas da eTFG dos três genótipos (SS, SL e LL) diferiram significativamente (p= 0,009), e quando LL foi comparado com LS + SS, a mediana de LL foi significativamente maior (p=0,005). Estes resultados sugerem que tanto o estado homozigótico TT do polimorfismo rs2071746, como os homozigotos LL das repetições (GT)n apresentam um risco maior de desenvolver complicações renais. Em relação ao gene *BMPR1B*, as frequências de homozigotos GG do polimorfismo rs17022863 e de homozigotos AA da mutação rs4331783 foram significativamente maiores nos pacientes do que nos controles saudáveis (p=0,002 e p=0,008,respectivamente), sugerindo que esses genótipos possam estar sob vigência de algum tipo de seleção, positiva ou negativa, na doença AF. No entanto, não foram detectadas associações significativas entre esses SNPs e os valores de eTFG no

grupo de pacientes aqui analisados. De nosso conhecimento, este é o primeiro estudo que investiga a associação entre polimorfismos nos genes *HMOX1* e *BMPR1B* e eTFG em pacientes brasileiros com AF. Nossos resultados provêem suporte adicional para o papel desses genes na nefropatia da AF e podem contribuir para melhores prevenção e acompanhamento desses pacientes.

Palavras-Chave: Anemia falciforme, Hemólise, Estresse oxidativo, Polimorfismos Genéticos, Taxa de filtração glomerular.

ABSTRACT

Oxidative stress caused by hemolysis is implicated in the pathophysiology of renal complications in sickle cell anemia (SCA). Mutations affecting genes involved in the oxidative and signalling pathways may be associated with kidney disease in SCA. We determined the allelic and genotypic frequencies of some common polymorphisms in the promoter regions of the Heme Oxygenase 1 (*HMOX1*) [SNP rs2071746 (A>T) and (GT)n repeats, short and long alleles] and Bone Morphogenetic Protein Receptor type 1 B (*BMPR1B*) [SNPs rs17022863 (A>G), rs4331783 (A>G) and rs1470409 (A>G)] genes in 75 SCA adult patients in a steady state and without hydroxyurea treatment, and compared them with those of 160 healthy controls from the same geographical region of the country and with the same ethnic characteristics as the patients. We also investigated whether these polymorphisms may influence the glomerular filtration rate estimated (eGFR) for these patients. The four SNPs were genotyped using TaqMan assays, while the *HMOX1* (GT)n repeats were determined by PCR fragment size analysis. The eGFR was calculated by Modification of Diet in Renal Disease (MDRD) formula. All the genotype frequencies were in Hardy-Weinberg equilibrium. Regarding *HMOX1* rs2071746, we found that the eGFR median was significantly higher in patients with the TT genotype (p=0.019), inclusive when TT was compared with AT+AA (p=0.009); in relation to the (GT)n repeats, the eGFR medians of the three genotypes (SS, SL and LL) significantly differed (p=0.009), and when LL was compared with LS+SS, the LL eGFR median was significantly higher (p=0.005). These results suggest that both the homozygous TT for rs2071746 and the homozygous LL for (GT)n repeats lead to a higher risk of developing renal complications. Concerning *BMPR1B*, the genotype frequencies of GG for rs17022863 and AA for rs4331783 were significantly higher in patients than in controls (p=0.002 and p=0.008, respectively), suggesting that these genotypes may be negatively or positively selected in SCA. However, we could not find any significant association between these SNPs and the eGFR in the group of patients studied here. To our knowledge, this is the first study investigating association between polymorphisms in the *HMOX1* and *BMPR1B* genes and eGFR in Brazilian SCA patients. Our results provide additional support for the role of these genes in SCA nephropathy and may contribute to prevention and better follow-up of these patients.

Keywords: Sickle Cell Anemia, Hemolysis, Oxidative stress, Genetic Polymorphisms, Glomerular Filtration Rate.

LIST OF ABREVIATIONS

- ATII: Angiotensin II
- ATRI: Angiotensin Receptor I
- BMPR1B: Bone Morphogenetic Protein Receptor type 1B
- CKD: Chronic Kidney Disease
- CO: Carbon Monoxide
- cGMP: cyclic Guanosine Monophosphate
- DNA: Deoxyribonucleic Acid
- Fe: Iron
- FSGS: Focal Segment Glomerulosclerosis
- eGFR: Estimated Glomerular Filtration Rate
- Hb: Hemoglobin
- HbA: Adult Hemoglobin
- HbS: Sickle Hemoglobin
- HbSS: Homozygous Sickle Hemoglobin
- HEMOPE: Hematology and Hemotherapy Center of Pernambuco, Recife
- HMOX: Heme Oxygenase
- NO: Nitric oxide
- RBC: Red blood cells
- ROS: Reactive Oxygen Species
- SCA: Sickle Cell Anemia
- SCN: Sickle Cell Nephropathy
- SCR: Serum creatinine
- sGC: soluble quanylyl cyclase

- TGFB1: Transforming growth factor beta 1
- VOC: Vaso-occlusive Crisis
- WBC: White blood cell

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1. INTRODUCTION

1.1 Sickle Cell Anemia

Sickle cell anemia (SCA) is a homozygous inheritance of abnormal sickle hemoglobin (HbS), a single β globin gene mutation (GAG>GTG) at the sixth codon of the human β globin gene *(HBBS),* on chromosome 11 specifically on the short arm position 15.5. This mutation occur as a result of substitution of glutamic acid for valine residue (glu6val) (HBB:c.20A>T; p.E7V) leading to polymerization of HbS molecules. During deoxygenation the HbS molecule polymerizes and this causes the erythrocytes to assume a sickle shape, the sickle cell becomes stiff and inflexible to pass through small capillaries [1,2]. SCA is characterized by chronic vaso-occlusion and severe hemolytic anemia associated with endothelial dysfunction. SCA is known to be clinically heterozygous despite the fact that all the affected patients are homozygous for gene β^S, with a wide range of clinical manifestations, including renal complications, acute chest syndrome, and leg ulceration [3,4].

The highest prevalence of HbS is found in Sub-Saharan African, and parts of the Mediterranean region, which include the Middle East and the Indian Subcontinent, although the β^S gene has spread around the world through population migration [5]. Sub-Saharan African record the highest percentage of 75% annual new births with SCA [6], and the demographic projections support the fact that this high prevalence is likely to grow further in coming decades [5-7]. It is generally believed that HbS was imported to Brazil mainly by slave trade during the colonial period and as a result of population migration [6,8,9]. In Brazil, the prevalence of the β^S allele ranges from 1.2% to 10%, depending on the region of the country [6]. The prevalence of HbS in Brazil in 2010 was 1 per 650, 1 per 1,200 and 1 per 4,000 live births in Bahia, Rio de Janeiro and Sao Paulo respectively [6].

1.2 Pathophysiology of Sickle Cell Anemia

SCA is characterized by a complex pathophysiology that leads to deformation of red blood cells (RBC) which affect the physical properties of RBC [10]. In SCA HbS polymerization is the essential pathophysiological occurrence which is highly dependent on the oxygen tension. The replacement of glutamic acid, a polar amino acid, by valine, a nonpolar amino acid at 6^{th} codon of the human beta globin gene on chromosome 11p 15.5, results in a marked decrease in the solubility of sickle hemoglobin in the deoxygenated state, leading to hemoglobin polymerization, tactoid formation, RBC rigidity and subsequent RBC membrane damage [10,11]. Change in shape of RBCs is initially reversible upon re-oxygenation; however, membrane damage occurs with each episode of sickling and eventually influx of calcium, with efflux of potassium and water. Hence the RBC eventually becomes irreversible sickle shape, the irreversible sickle RBC becomes stiffer with increased amount of HbS polymer and hemolysis is associated with irreversible structural changes of sickle RBCs [12]. The Gardos channel is a K^+ efflux channel activated by the intracellular Ca^{++} increase due to deoxygenation. Thus, K^+ output results in water efflux, cell dehydration and increased internal HbS concentration [13]. This leads to cellular dehydration, hemolysis and nitric oxide (NO) depletion [14]. Patients are therefore susceptible to vaso-occlusion and subsequent organ damage [15]. In addition to episodic cycles of ischemia reperfusion injury and subsequent organ damage, abnormal adhesion of leukocytes and platelets, coagulation, inflammation and hypoxia are some of the characteristics features of SCA. These manifestations are brought about through two major pathways: vaso-occlussion with ischemia-reperfusion injury and hemolytic anemia [16], as shown in Figure 1.

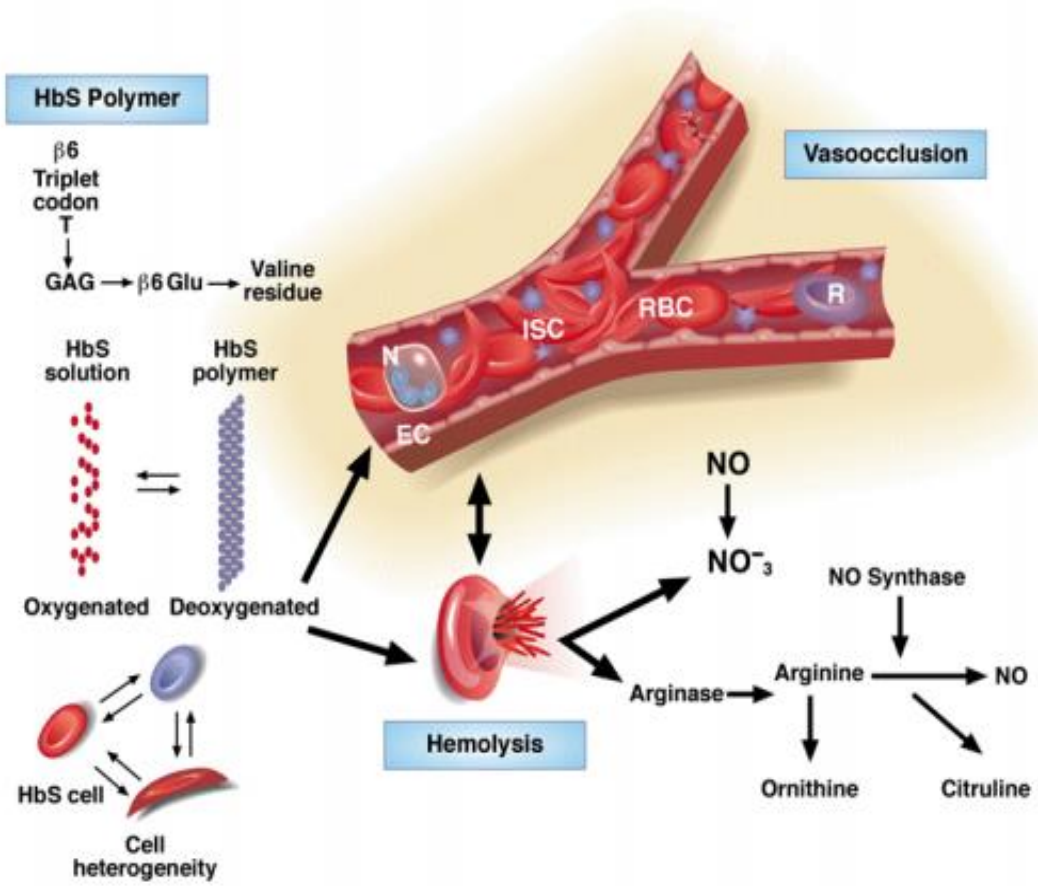

Figure 1: The alteration in the β globin chain leads to the replacement of glutamic acid for valine, resulting in the polymerization of hemoglobin (Hb) molecule when deoxygenated. The sickle polymer injuries the erythrocytes and eventually produces irreversible membrane damage (Steinberg *et al.* 2008).

1.3 Hemolysis in Sickle Cell Anemia

Hemolysis is a common pathophysiologic process in SCA. Erythrocytes injury leads to extra and intravascular hemolysis, endothelial dysfunction, vasculopathy, and occlusion of small and large blood vessels, in extra-vascular hemolysis where red cells are damaged by macrophages in the spleen, liver, bone marrow and reticulo-endothelial system [17], while intravascular hemolysis is a potential cause of oxidative injury and endothelial damage in SCA, both hemolysis produces tissue ischemia/reperfusion injury and inflammation. Sickle erythrocyte has high tendency to hemolyse due to it unstable nature of HbS. This therefore, shorten the lifespan of sickle erythrocyte [17]. When Hb is released from RBC due to hemolysis, the free Hb and arginase are liberated from the RBC which set in motion a cascade of molecular events that damage vascular endothelium, the arginase which destroys arginine leading to nitric oxide (NO) depletion and vascular dysfunction and may trigger oxidative tissue damage [17,18].Oxidation of ferrous to Ferric Hb (Fe^{3+}), which generates hydroxyl and lipid peroxyl radicals, promotes vascular and renal injury, which may result to pulmonary hypertension and chronic kidney disease as patients age[19]. Oxidation of hemoglobin can result in the release of free heme into plasma which in excess triggers inflammation, vaso-occlusion and coagulation, free heme is removed by hemopexin [17].

Free heme released from oxidized Hb induces vascular inflammation and is a major source of oxidative stress among patients with SCA, where continuous auto-oxidation of extracellular Hb produces superoxide which dismutates into hydrogen peroxide [17]. Thus, the level of oxidative stress is higher in SCA patients when compared with healthy individuals [20], and free hemoglobin is found to be increased by 10 fold in SCA [21], with

an average concentrations of 3.5uM (range 0.4-10.9uM) at steady state and 5.3uM (1.0-25.3uM) during severe hemolysis [22-23].

1.4 Renal Pathophysiology/Hyperfiltration in SCA

SCA associated nephropathy is a growing matter of concern, because renal failure affects 12% to 21% of adult SCA patients [24,25] and up to 80% of aging SCA patients [26]. Studies have shown that approximately 16-18% of overall mortality in SCA patients are associated with kidney disease [27,28]. SCA patients exhibit episodes of hematuria and tubular abnormalities as a consequential effect of hemolysis and some of the early clinical manifestations [19,26,28], including hyperfiltration, hypertrophy, and impaired urinary concentrating ability are described as early as in infancy in SCA patients [28]. These are associated with increased renal plasma flow, also result to an increased glomerular filtration rate (GFR) [29]. Renal complication in SCA is characterized by alterations in renal hemodynamics. Higher GFR is mediated by increased generation of nitric oxide as well as an increased production of renal kallikrein and other vasoactive kinins [30]. In a study using animal model, it was observed that some nephrons were hypertrophic and associated with increased glomerular blood flow, and the afferent arteriolar resistance decreases more than the efferent arteriolar resistance which then progresses to kidney disease [31]. Consequently, intraglomerular hypertension occurs. Glomerular hypertension has been shown in some experimental studies to mediate progressive kidney damage following a variety of initiating injuries [31]. Hemolysis is a risk factor for hyperfiltration and proteinuria associated with increased GFR among patients with SCA [28,32,33].

Therefore, mutations in genes involved in oxidative and signalling pathways may influence renal function in SCA patients.

1.5 *HMOX1* Gene

This gene located on the chromosome 22q12.3 is important gene because of its involvement in the oxidative pathway and its protein induced by heme during hemolysis. There are two isoforms of Heme oxygenase enzyme (HMOX): the heme oxygenase -1 (HMOX1) is the inducible isoform while heme oxygenase 2 (HMOX2) is the constitutive isoform [34]. The inducible isoform, heme oxygenase-1 (HMOX1), is expressed in various tissues, including liver, spleen, lung, and brain and is up-regulated by stimuli, such as heme, oxidants hypoxia, oxidative stress and certain cytokines [34]. Oxidative stress particularly, appears to be a major factor in HMOX1 induction under pathologic conditions [35,36]. It plays a role in the regulation of reactive oxygen species (ROS) mediated pathway. HMOX1 catalyses the conversion of heme to biliverdin, carbon monoxide (CO) and iron (Fe), biliverdin reductase then converts biliverdin to bilirubin as shown in the Figure 2. Both biliverdin and bilirubin have been known to act as scavengers of ROS, and carbon monoxide has anti-inflammatory and anti-oxidant effects [37]. HMOX1 is thus known as an oxidative stress responsive protein that is usually stimulated by multiple stimuli and has been proposed to provide an important cellular response that protects cells against oxidative damage. The importance of *HMOX1* gene was reported using (*Hmox1*$^{-1-}$) deficient mice which confirmed that this gene is indispensable to survival and in particular, to protect the mice from oxidative stress [38,39]. Another important study revealing the importance of this gene was carried out by Shiraishi *et al.* (2000) with a Hmox1 knock out mice (*Hmox1*$^{-1-}$) [40], where Hmox1 deficient mice was found with renal injury, severe irreversible renal failure and 100% death. This expresses the importance of this gene to oxidative injury and its association with marked cytoprotection. In a similar experiment, Nath *et al.*,(2000) observed the contrary effect in (*Hmox1*$^{+1+}$) mice that displayed mild, reversible renal injury, and 0% death [41]. Molecular genetics of the *HMOX1* gene provided additional support for the protection of the kidney against oxidative stress [42]. Modulated

by several identified functional polymorphisms in this gene, humans differ quantitatively in their ability to mount HMOX1 response. Investigations into some of the beneficial effects of HMOX1 bye products such as CO revealed that this molecule exerts vasodilatory effects through cyclic guanosine monophosphate (cGMP) dependent smooth muscle relaxation similar to the well-established vasodilator nitric oxide (NO), CO binds to the heme moiety of soluble guanylyl cyclase (sGC), causing activation of cGMP and resultant vascular relaxation [43]. A number of studies have examined DNA polymorphisms in the *HMOX1* gene that might influence the level of heme oxygenase response [44,19].

There are two most studied promoter variants [a -413A>T(rs2071746) single-nucleotide polymorphism (SNP) and (GT)n micro satellite polymorphism] several studies have reported their functional roles in regulation of heme oxygenase levels [45,46]. When stimulated by hydrogen peroxide in vitro, short repeats alleles (<25 repeats depending on the cut of the study) show increased promoter activity compared with longer repeats alleles (> 25 repeats) [47]. The *HMOX1* (GT)n tandem repeats polymorphism has been previously shown to correlate with HMOX1 activity [46]. The (GT)n repeats is highly polymorphic and accumulating evidence has suggested that persons with lower numbers of repeats might have higher inducible heme oxygenase expression [48,49].

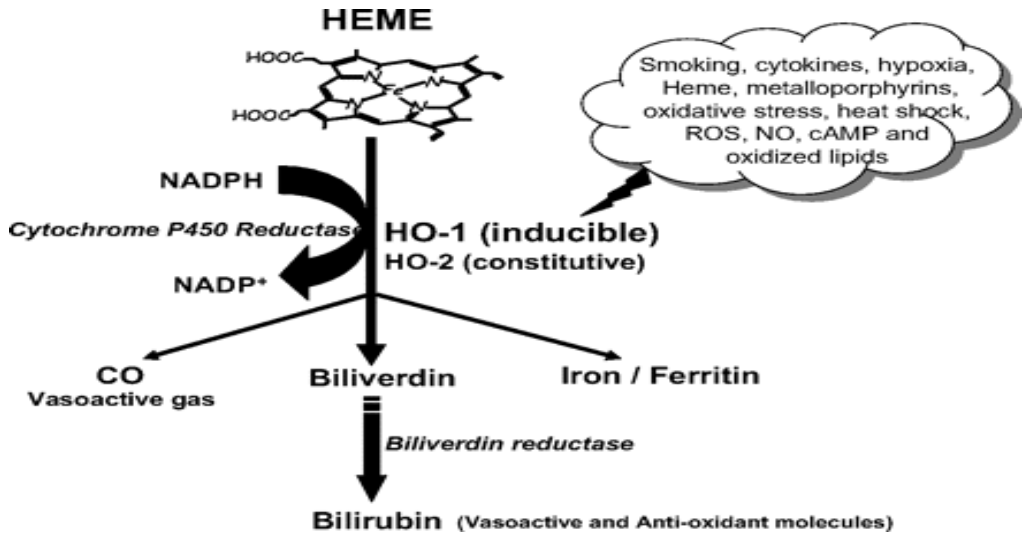

Figure 2: Role of heme oxygenase-1 and the actions of its products in response to oxidative stress in SCA. Heme oxygenase-1 degrade free heme to biliverdin, iron and carbon monoxide (Abraham and Kappas, 2008)

1.6 *BMPR1B* Gene

BMPR1B gene located on chromosome 4q22.3 encodes a member of the bone morphogenetic protein (BMP) receptor family and a member of transforming growth factor beta (TGFB) superfamily which are large group of structurally related cell regulatory proteins [50]. BMP/TGFB initiate signalling across the plasma membrane into the cell by inducing heteromeric complexes of type l and type ll receptors with serine/threonine kinase activity [51]. Studies have shown that BMP/TGFB signalling molecules regulate cell proliferation, differentiation, apoptosis and adhesion of many different cell types [50.52,53].Patel *et al*. (2005) observed that SNPs in the *BMPR1B* gene were associated with nephropathy in Type 1 diabetes [54]. Other studies have also revealed that diabetic nephropathy patients share some common features with sickle cell nephropathy, such as glomerular hyperfiltration, mesangial cell proliferation and mesangial expansion [54,55]. Nolan *et al*.(2007) studying SCA patients, found that the SNP rs17022863, and the combination of SNPs rs17022863, rs4331783 and rs1470409 in haplotypes, are significantly associated with the estimated GFR (eGFR) [55].

Considering the high prevalence rates of mortality in SCA (16-18%) as a result of kidney disease (27,28), genetic variation studies play a vital role in gaining a full understanding of renal complications. Hence, SNPs and (GT)n repeats of *HMOX1* and SNPs of *BMPR1B* are believed to be involved in oxidative/signalling pathways which may influence renal function in SCA. However, there are few studies on mutations in *HMOX1* and *BMPR1B* and their association with eGFR and, to our knowledge, none in Brazilian Sickle Cell Patients.

Therefore, some mutations in these genes (*HMOX1* and *BMPR1B*) which may influence renal function in SCA and the full knowledge about the influence of these genetic variants is still not clear. In order to investigate if common polymorphisms in *HMOX1* and *BMPR1B* genes might modulate renal function in SCA patients, we determined the allelic and genotypic frequencies of rs2071746 and (GT)n repeats in *HMOX1* and rs17022863, rs4331783, rs1470409 in *BMPR1B* in SCA patients and controls. We also investigated the association between these mutations and eGFR in the group of patients.

2. OBJECTIVES

General:

- To investigate the association of common SNPs and (GT)n repeats in *HMOX1* and *BMPR1B* genes with estimated glomerular filtration rate (eGFR) in Brazilian patients with SCA.

Specific:

- To determine the allelic and genotypic frequencies of rs2071746 and (GT)n repeats of *HMOX1* and rs17022863, rs1470409, rs4331783 of *BMPR1B* in SCA patients and healthy controls (HbAA).
- To investigate their influence on the eGFR of these patients.

3. MATERIALS AND METHODS

3.1 Study participants

The study comprised 75 SCA patients (HbSS, 28.3±8.2 years old, 54.7% males) on follow up at the Hematology Center of Pernambuco (HEMOPE), in Recife, state of Pernambuco, northeast of Brazil. The control group was composed of 160 healthy adults (blood donors) (Hb AA, 40±10.1 years old, 78% males) from the same Brazilian region and with ethnic characteristics similar to those of the patients. Further information on the biodata was obtained by interview and from patient's medical records. The local ethics committee approved this study, and informed consent was obtained from all participants, according to the Helsinki Declaration.

3.2 SNP Genotyping

The identification of the *HMOX1* -413A>T (rs2071746) and *BMPR1* SNPs (rs1702283, rs14070409, rs4331783) were performed by TaqMan® SNP genotyping assay according to the manufacturers protocol. The SNP genotyping assays used for the experiments were presented in the Table 1 (Applied Biosystems, Foster city, CA, USA). The final reaction volume was optimized to 5ul reaction containing final reaction concentration of both the working stock assay and TaqMan Universal PCR master mix 75ng of template DNA was added to each reaction. The reactions were performed in 96 well. TaqMan real time PCR was performed and two no DNA template control (NTCs). Initial denaturation step of 95^{o}C for 10minutes, which was succeeded by 50 cycles denaturation, each containing sub steps of fragment denaturation, primer annealing and primer extension at 95^{o}C for 15seconds,60^{o}C for 29seconds and 60^{o}C for 1minute respectively. After PCR amplification, fluorescence signals were analysed on a Step One Plus Real Time PCR System (Applied Biosystems, Foster city, CA, USA). Genotypes were determined by manually reviewing each allelic discrimination plot with

sequence Detection system version 2.3 software (Applied Biosystems, Foster city, CA, USA).

Table1: SNP genotyping assays used for experiments

Gene	SNPs	Context Sequence [VIC/FAM]
HMOX1	rs2071746	AGTTCCTGATGTTGCCCACCAGGCT[A/T] TTGCTCTGAGCAGCAGCGCTGCCTCCCA
BMPR1B	rs1470409	CAGACTCTGTGACTTGGCCTCCTGT[A/G]TAAATCTCGTCC CAGTACTTTGCA
	rs4331783	ATTCTGCCACAAACATTCATTCA[A/G]TAGAACATGGCACA TAGTCCTGGAG
	rs17022863	GCATTTTAGTCACTGGATTATTACCTGG[A/G]TTTTTAAGTA TTTTGCATCCCTGTG

3.3 (GT)n Repeats Genotyping

The rs3074372 identification was performed by PCR using a forward primer 5'- (6-FAM) labelled (*) for detection by fragment analysis in capillary electrophoresis system. The PCR reaction was prepared in 30 µL volume with 150ng of genomic DNA, 1X Reaction Buffer (BIOTOOLS B&M Labs, Valle de Tobalina, Madrid, Spain), 2.16mM $MgCl_2$, 1.33 mM of dNTP mix, 133 nM of each primer [named HMOX1_prom_*F (AGAGCCTGCAGCTTCTCAGA) and HMOX1_prom_R (ACAAAGTCTGGCCATAGGAC)] (21) and 1 U Taq DNA Polymerase (BIOTOOLS B&M Labs Valle de Tobalina, Madrid, Spain). Thermal cycle conditions were as follows: preheating at 96°C by 2 minutes, followed by 25 cycles of 96°C for 30 seconds, 60°C for

40 seconds, and 72°C for 40 seconds. An ended step at 72°C for 30 min was performed to promote adenylation of the PCR products. The PCR product (1 µL) was added to 8.7 µL Hi-Di Formamide (Applied Biosystems, Carlsbad, CA, USA) and 0.3 µL of a GeneScan™ 500 LIZ™ size standard (Applied Biosystems, Carlsbad, CA, USA) and the fragments ranged from 92 - 160 bp, corresponding to 12 - 46 fragment repeats, respectively, were separated by capillary electrophoresis on a ABI 3500 Genetic Analyzer, analysed by Gene Mapper v4.1 Software (both Applied Biosystems, Carlsbad, CA, USA) and classified according to Bean *et al.*(2012) as short (S), with ≤ 25 repeats, and long (L), with > 25 repeats [56].

3.4 The estimated Glomerular Filtration Rate (eGFR)

The serum creatinine levels and the eGFR of these SCA patients were previously obtained [57]. Estimated Glomerular Filtration Rates were determined by Modification of Diet in Renal Disease (MDRD):

eGFR(mL/min/1.73m^2)= 175 x (SCR mg/dL)-1.154 x (Age) -0.203 x (0.742) if female (58).

3.5 Statistical Analysis

Statistical analyses were performed using Statistical Analysis System (SAS) version 9.4 (SAS Institute Inc. Cary, USA). Data were reported as Means, Percentages and Standard deviations. Gender and Age were adjusted using logistic regression. Hardy-Weinberg Equilibrium (HWE) was checked by chi square test. Mann-Whitney test was used to compare two variables. One-way ANOVA was used to compare three variables. The $p<0.05$ value were considered statistical significance.

4. MANUSCRIPT

Polymorphisms in the Heme Oxygenase-1 and Bone Morphogenetic Protein Receptor Type 1B Genes and Estimated Glomerular Filtration Rate in Brazilian Sickle Cell Anemia Patients

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ABSTRACT

Oxidative stress caused by hemolysis is implicated in the pathophysiology of renal complications in sickle cell anemia (SCA). Mutations affecting genes involved in the oxidative and signalling pathways may be associated with kidney disease in SCA. We determined the allelic and genotypic frequencies of some common polymorphisms in the promoter regions of the Heme Oxygenase 1 (*HMOX1*) [SNP rs2071746 (A>T) and (GT)n repeats, short and long alleles] and Bone Morphogenetic Protein Receptor type 1 B (*BMPR1B*) [SNPs rs17022863 (A>G), rs4331783 (A>G) and rs1470409 (A>G)] genes in 75 SCA adult patients in a steady state and without hydroxyurea treatment, and compared them with those of 160 healthy controls from the same geographical region of the country and with the same ethnic characteristics as the patients. We also investigated whether these polymorphisms may influence the glomerular filtration rate estimated (eGFR) for these patients. The four SNPs were genotyped using TaqMan assays, while the *HMOX1* (GT)n repeats were determined by PCR fragment size analysis. The eGFR was calculated by Modification of Diet in Renal Disease (MDRD) formula. All the genotype frequencies were in Hardy-Weinberg equilibrium. Regarding *HMOX1* rs2071746, we found that the eGFR median was significantly higher in patients with the TT genotype (p=0.019), inclusive when TT was compared with AT+AA (p=0.009); in relation to the (GT)n repeats, the eGFR medians of the three genotypes (SS, SL and LL) significantly differed (p=0.009), and when LL was compared with LS+SS, the LL eGFR median was significantly higher (p=0.005). These results suggest that both the homozygous TT for rs2071746 and the homozygous LL for (GT)n repeats lead to a higher risk of developing renal complications. Concerning *BMPR1B*, the genotype frequencies of GG for rs17022863 and AA for rs4331783 were significantly higher in patients than in controls (p=0.002 and p=0.008, respectively), suggesting that these genotypes may be negatively or positively selected in SCA. However, we could not find any significant association between these SNPs and the eGFR in the group of patients studied here. To our knowledge, this is the first study investigating association between polymorphisms in the *HMOX1* and *BMPR1B* genes and eGFR in Brazilian SCA patients. Our results provide additional support for the role of these genes in SCA nephropathy and may contribute to prevention and better follow-up of these patients.

Keywords: Sickle Cell Anemia, Hemolysis, Oxidative stress, Genetic Polymorphisms, Glomerular Filtration Rate.

INTRODUCTION

Sickle cell anemia (SCA) is a chronic and severe hemolytic anemia associated with endothelial dysfunction, vaso-occlusion and inflammation. It is caused by a homozygous inheritance of a single point mutation (GAG>GTG) at the 6th codon of the β globin gene, on 11p15.5, resulting in the replacement of glutamic acid for valine at the 6th position of the β globin chain and leading to the formation of hemoglobin S (HbS) (HBB:c.20A>T; p.E7V). Deoxy-HbS polymerizes inside the erythrocytes resulting in the formation of sickled red blood cells, related to the hemolysis and vaso-occlusive events, organ damage and a wide range of clinical manifestations [1-4].

One of the most important clinical complications in SCA is kidney disease, responsible for 15-18% of the mortality rate in adult patients [3,4]. In the early stages, it includes glomerular hyperfiltration, glomerular enlargement, and hematuria [5-7], progressing to chronic kidney disease (CKD) [8]. Hemolysis seems to be related to the pathogenesis of renal disease in SCA [9-11]. Chronic hemolysis results in high renal plasma flow which can lead to endothelial dysfunction causing hemodynamic changes that result to renal functional and structural abnormalities and, consequently, increase in glomerular filtration rate (GFR) [6,7,11]. Genetic variants of genes related to oxidative and signalling pathways, as the Heme Oxygenase-1 (*HMOX1)* and Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*) genes, respectively, have been associated with some of the clinical complications in SCA, such as stroke, osteonecrosis and acute chest pain [12-15]. Studies have revealed that in these patients the release of free heme triggers the formation of reactive oxygen species (ROS) and oxidative stress, induces HMOX1 activity and increases the conversion of oxidized angiotensinogen to

angiotensin II, which mediates the generation of signalling molecules, capable of inducing systemic responses leading to renal damage [16-18].

HMOX1 is the rate limiting enzyme that degrades heme through oxidation to yield equimolar quantities of biliverdin, carbon monoxide (CO) and free iron (Fe) [19]. A severe, irreversible renal failure with 100% death was found in mice deficient Hmox1 (-/-) in contrast with Hmox1 (+/+) mice, that showed mild, reversible renal injury and 0% mortality [20]. *HMOX1* gene has two promoter variants that have been widely studied, a -413 A>T single nucleotide polymorphism (SNP) (rs2071746) and a (GT)n microsatellite polymorphism [21]. Short repeats (n≤25 or n<27, according to the cut off of different studies) have been associated with higher gene expression levels than the long (GT)n repeats (n≥25 or n≥27) [21].

BMPR1B gene encodes a member of the bone morphogenetic protein (BMP) receptor family of transmembrane serine/threonine kinases**.** The ligands of this receptor are BMPs, which are members of the TGF-beta superfamily. BMPs are involved in endochondral bone formation and embryogenesis. These proteins transduce their signals through the formation of heteromeric complexes of two different types of serine (threonine) kinase receptors: type I receptors of about 50-55 kD and type II receptors of about 70-80 kD [22]. Patel *et al*. observed that some SNPs in the *BMPR1B* gene are associated with nephropathy in Type 1 diabetes [23]; Nolan *et al*., studying SCA patients, found that the SNP rs17022863, and the combination of SNPs rs17022863, rs4331783 and rs1470409 in haplotypes, are significantly associated with the estimated GFR (eGFR) [24].

In Brazil the prevalence of the β^s allele is high, varying from 1.2% to 10.9% depending on the region of the country [4]. To our knowledge, there are no studies evaluating the influence of polymorphisms in the *HMOX1* and *BMPR1B* genes on the renal function in Brazilian SCA patients. Thus, the aim of this study was to determine and compare the

allelic and genotypic frequencies of the SNP rs2071746 and (GT)n repeats in the *HMOX1* gene and the SNPs rs17022863, rs4331783 and rs1470409 in the *BMPR1B* gene between Brazilian adult SCA patients and healthy controls and investigate whether these polymorphisms may influence the eGFR of these patients.

MATERIALS AND METHODS

The study comprised 75 SCA patients (HbSS, 28.3±8.2 years old, 54.7% males) on follow up at the Hematology and Hemotherapy Center of Pernambuco (HEMOPE), in Recife, state of Pernambuco, northeast of Brazil. The patients were in a steady state and without hydroxyurea treatment for at least three months. The control group was composed of160 healthy adults (blood donors) (Hb AA, 40±10.1 years old, 78% males) from the same Brazilian region, with ethnic characteristics similar to those of the patients. Further information on the biodata was obtained by interview and from patient's medical records. The local ethics committee approved this study, and informed consent was obtained from all participants, according to the Helsinki Declaration.

Genomic DNA from all patients and controls was extracted from peripheral blood (25). The SNPs were genotyped by TaqMan® SNP Genotyping Assay in the Step One Plus Real-Time PCR system according to the manufacturer protocol (Applied Biosystems, Foster city, CA, USA). The (GT)n repeats were identified by capillary electrophoresis on a ABI3500 Genetic Analyzer, using the Gene Mapper v 4.1 Software (both Applied Biosystems, Carlsbad, CA, USA). They were classified according to Bean *et al.,* 2012 as short (S) with ≤ 25 repeats and long (L) with > 25 repeats [15].

The eGFR was determined in the SCA patients by using the simplified prediction equation MDRD (Modification Diet in Renal Disease): eGFR (mL/min/1.73m^2) = 175 x (serum creatinine mg/dL)$^{-1.154}$ x (age)$^{-0.203}$ x 0.742 (if female) (9).

Statistical Analysis System (SAS) for Windows version 9.4 (SAS Institute Inc, Cary, U.S.A) was used for the analysis. Allelic and genotypic frequencies were determined in both, patients and controls. Hardy-Weinberg Equilibrium (HWE) was checked by X^2 test. Age and gender were adjusted using regression logistics. One way ANOVA was used to compare three variables, while Mann-Whitney was used to compare two variables against eGFR in SCA patients. The *p* values <0.05 were considered statistically significant.

RESULTS

The allelic and genotypic frequencies found for rs2071746 are presented in Table 1,(Appendix ; Figure 1) while those found for the (GT)n repeats in the *HMOX1* gene are shown in Table 2 (Appendix; Figure 2).

Table 1. Allelic and genotypic frequencies for rs2071746 in the *HMOX1* gene

SNPs	**Alleles/ Genotypes**	**SCA Patients n=74**	**Controls n=131**	***p*-values**	**Median eGFR (mL/min/1.73m^2)**	***p*-values eGFR**
	A	48.0%	55.0%			
	T	52.0%	45.0%			
HMOX1	AA	18 (24.3%)	37 (28.2%)		156.22	
rs2071746	AT	33 (48.6%)	69 (52.7%)	0.39	161.46	**0.019**
	TT	20 (27.0%)	25 (19.1%)		188.39	
	TT	20 (27%)	25 (19.1%)		188.39	
	AT+AA	54 (73%)	106 (80.9%)		160.86	**0.009**
HWE p-value		0.82	0.47			

HWE: Hardy-Weinberg equilibrium, n: number, SNP: Single Nucleotide Polymorphism, eGFR; estimated Glomerular Filtration Rate

Table 2: Distribution of genotype frequencies of the (GT)n repeats in the *HMOX1* gene

Genotypes	**SCA patients n=75**	**Controls n=160**	***p*-values**	**Median eGFR (mL/min/1.73m^2)**	***p*-values eGFR**
LL	42 (56.0%)	85 (53.1%)		173.83	
SL	28 (37.3%)	64 (40.0%)	0.92	143.20	**0.009**
SS	5 (6.7%)	11 (6.9%)		193.94	
SL+LL	70 (93.3%)	149 (93.1%)		163.63	
SS	5 (6.7%)	11 (6.9%)	0.95	193.94	0.74
LL	42 (56.0%)	85 (53.1%)		173.83	
SL+SS	33 (44.0%)	75 (46.9%)	0.68	147.52	**0.005**
HWE *p*-value	0.91	0.82			

HWE: Hardy-Weinberg equilibrium, n: number, L: Long, S: Short, eGFR: estimated glomerular filtration rate

The allelic and genotypic frequencies for rs17022863, rs4331783 and rs1470409 in the *BMPR1B* gene in SCA patients and controls are presented in Table 3 (Appendix ;Figure 3).

Table 3: Allelic and Genotypic frequencies for rs17022863, rs4331783 and rs1470409 in the *BMPR1B* gene

SNPs	**Allele/ Genotypes**	**SCA Patients n=75**	**Controls n=132**	***p*-values**	**Median eGFR (mL/min/1.73m^2)**	***p*-values eGFR**
	A	59.0%	72.0%			
	G	41.0%	28.0%			
BMPR1B	AA	27 (36.0%)	66 (50.8%)		166.61	
rs17022863	AG	35 (46.7%)	56 (43.1%)	**0.002**	161.62	0.87
	GG	13 (17.3%)	8 (6.2%)		192.94	
p-value (HWE)		0.77	0.38			
	A	44.0%	45.0%			
	G	56.0%	55.0%			
BMPR1B	AA	13 (17.3%)	27 (21.3%)		180.06	
rs1470409	AG	40 (53.3%)	63 (49.6%)	0.16	161.38	0.94
	GG	22 (29.3%)	37 (29.1%)		168.90	
p-value (HWE)		0.47	0.98			
	A	44.0%	69.0%			
	G	56.0%	31.0%			

BMPR1B	AA	13 (18.0%)	8 (6.1%)		185.36	
rs4331783	AG	37 (51.4%)	65 (49.2%)		161.29	0.83
	GG	22 (30.5%)	59 (44.7%)	**0.008**	166.13	
p-value (HWE)		0.71	0.070			

HWE: Hardy-Weinberg equilibrium, n: number, SNP: Single Nucleotide Polymorphism, eGFR: estimated Glomerular Filtration Rate

Discussion

HMOX1 enzymes have been known for its anti-oxidant and anti-inflammatory activities [26-28]. In SCA, these enzymes are particularly important to protect patients from the damaging effects of excess heme released by high rates of chronic intravascular hemolysis [28]. A recent study with SCA mice has shown that induction of HMOX1 protein significantly inhibits inflammatory markers and vaso-occlusion [29]. We determined the allelic and genotypic frequencies of two common polymorphisms in the promoter region of the *HMOX1* gene in Brazilian adult SCA patients and compared them with those found in a control group. The frequencies were all in HWE. We also investigated whether the different genotypes related to these two polymorphisms could influence the eGFR of our patients. Regarding rs2071746, we observed that the eGFR median was significantly higher in patients with the TT genotype (p=0.019), inclusive when TT was compared with AT+AA (p=0.009). This finding is in agreement with previous studies which found association of the T allele with reduction of *HMOX1* gene expression [15,30] and suggests that patients with the TT genotype are at higher risk of developing renal complications. In relation to the (GT)n repeats, the eGFR medians of the three genotypes (SS, SL and LL) significantly differed (p=0.009); when LL was compared with LS+SS, the LL eGFR median was significantly higher (p=0.005). This result also suggests that patients with the LL genotype may have higher risk of renal disease progression, supporting the concept that long alleles are associated with decreased HMOX1 activity, as found by Chen *et al* in patients with coronary artery disease [31].

The *BMPR1B* gene plays an important role in cell proliferation, inflammatory response and tissue repair. In our patients and controls, the allelic and genotypic frequencies of the SNPs rs17022863, rs4331783 and rs14070409 were all in HWE**;** statistical differences

between them were found for two SNPs, rs17022863 and rs4331783 (p=0.002 and 0.008, respectively), suggesting that they are being negatively or positively selected in SCA. However, we could not find any significant association of the different genotypes with eGFR, in contrast to Nolan findings, as previously commented [24]. Okocha *et al*. (2010) [32], investigating other polymorphisms in the same gene, and Saraf *et al.* (2015) [18], analysing four polymorphisms, three of which we analysed, also we could not observe association among the different genotypes and eGFR in SCA patients. The differences commonly observed in different studies may be due to different composition of the populations and/or sample sizes [33,34].

To our knowledge, this is the first study determining the allelic and genotypic frequencies of common polymorphisms in the *HMOX1* and *BMPR1B* genes and investigating their association with eGFR in Brazilian SCA patients. Our results showed that the homozygous TT for rs2071746 and homozygous LL for (GT)n repeats in the promoter region of *HMOX1* were associated with significantly higher eGFR medians, providing additional support for the role of *HMOX1* in renal complications in SCA patients. These results may contribute to prevention measures and better follow-up of our patients and, with other studies, allow a better understanding of the genetic factors related to the development of renal complications in SCA. We could not find any association among the SNPs rs17022863, rs4331783 and rs14070409 in the promoter region of the *BMPR1B* genes and eGFR in the group of patients studied here.

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5. DISCUSSION

Intravascular hemolysis is a potential cause of oxidative injury and endothelial dysfunction in SCA [33]. Human proximal tubular cell exposed to free hemoglobin has increased tendency of kidney injury, reduced viability and induction of HMOX1 due to hemolysis [33,34]. HMOX1 enzyme, is induced in response to oxidative stress function as cytoprotective protein in SCA patients from cytotoxic effect of heme, which are released by chronic intravascular hemolysis [59]. Studies revealed that SNPs in *HMOX1* are associated with various disease conditions including renal complications [56,60].Therefore SNPs found in *HMOX1* gene may modulate renal function in SCA patients. In this study we determined the allelic and genotypic frequencies of SNPs in the promoter region of *HMOX1* gene in Brazilian adult SCA patients and compared them with that of the control group. We also investigated if the different genotypes of these SNPs could influence the eGFR of the patients. The genotypic frequencies were in the Hardly-Weinberg equilibrium (HWE) and no difference between the patients and the controls. On *HMOX1* rs2071746 we did not observe a significant differences in the genotype frequencies when comparing patients and controls (p=0.38). The medians of the three genotypes AA, AT and TT showed significant difference among SCA patients (p=0.019). Regarding the association of the different genotypes SCA patients carrying the homozygous TT genotype had significant higher levels of eGFR when compared with other genotypes (p=0.019). In a study it was reported that the T allele in *HMOX1* rs2071746 was significantly associated with reduced HMOX1 activity [45]. This then suggest that these patients are at risk of developing CKD, the possible explanation may be the genetic variants decreases the regulation of HMOX1 protein and therefore less protein may be available and greater concentration of heme will increase in circulation.

The (GT)n repeats, the eGFR medians of the three genotypes (SS,SL and LL) significantly differed (p=0.009) when LL was compared with LS + SS, the LL eGFR median

was significantly higher (*p*=0.005). A hypothesis to explain these findings is that the homozygous genotype LL may be associated with reduced levels of HMOX1 activity there by exposing the patient to high risk of oxidative stress which may progress to CKD, a similar observation was made by Chen *et al.,* where they reported the (GT)n long repeats in *HMOX1* was associated with CKD among patients with coronary artery disease [61]. In a similar report made by Saraf *et al*.(2015) but in a different *HMOX1* SNPs rs743811 where long (GT)n repeats were associated with CKD in 247 adults African Americans SCA patients[60]. Furthermore, Bean *et al*.(2012) who observed that African American children with SCA, with short (GT)n repeats were associated with lower rates of hospitalization for acute chest syndrome [56].

All the SNPs studied here in *BMPR1* gene (rs17022863, rs4331783 and rs14070409); the allelic and genotypic frequencies were in HWE and did not significantly differ between patients and controls. Our result of SCA patients did not reveal any association with the eGFR, in contrast with the findings by Nolan *et al.* (2007), who found that SNPs in *BMPR1B* gene were associated with eGFR in SCA patients [55]. The differences observed between our study and that of Nolan and colleagues who researched on the same SNPs with our candidate SNPs may be due to different composition of the population and sample size. However, the distribution of genotype frequencies for rs17022863 and rs4331783 were significantly different between SCA patients and healthy controls, with (*p*=0.002) and (*p*=0.008) respectively. These differences may be as a result of natural selection in favour or against one of the two alleles compared, but possibly regarding some other clinical manifestation or complication [62,63].

6. CONCLUSION

To our knowledge, this is the first study investigating association of genetic variants of *HMOX1/BMPR1B* genes with eGFR in adult Brazilian SCA patients. Hence, our results revealed that homozygous TT and homozygous LL genotypes in the promoter region of SNP *HMOX1* rs 2071746 were significantly associated with eGFR in adult Brazilian SCA patients, both genotypes are associated with significant higher eGFR medians. Our results further reaffirmed the important role played by *HMOX1* as an antioxidant in the oxidative pathway. Further studies is recommended which would further validate our findings for better understanding of renal complication influenced by genetic variants. Early identification of high risk SCA patients, can contribute to a better management of SCA patients. All three SNPs rs17022863, rs4331783 and rs14070409 in *BMPR1* did not show any association with eGFR in our SCA patients.

Limitation of the study: the samples size in our study group were small.

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8. Appendix 1

Graphical representation

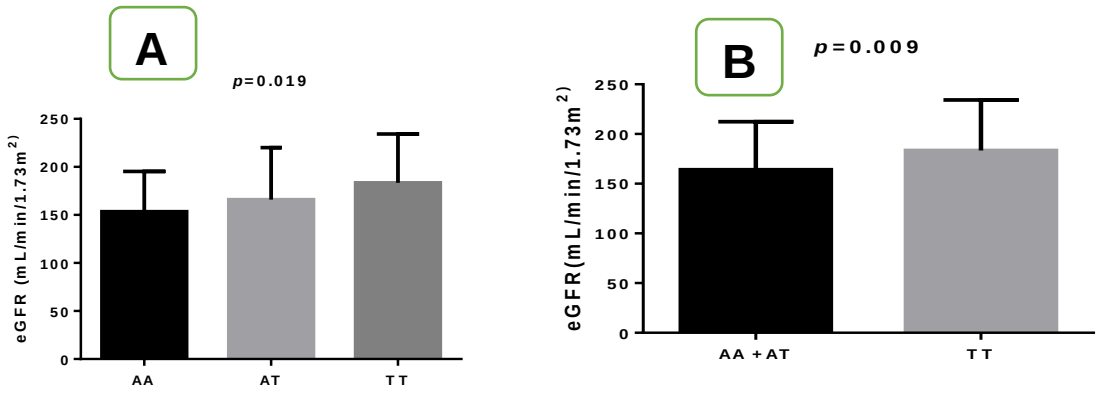

Figure 1: Estimation of the glomerular filtration rate (mL/min/1.73m^2).Calculated by the MDRD Method, in patients with sickle cell anemia according to the rs2071746 polymorphism of HMOX1. The X axis represents the HMOX1 genotype and the Y axis to eGFR (Statistical test used, n=75).The values are represented by means ± standard deviation. (A) HMOX1 rs 2071746 (*p*=0.019) (B) HMOX1 rs 2071746 (*p*=0.009)

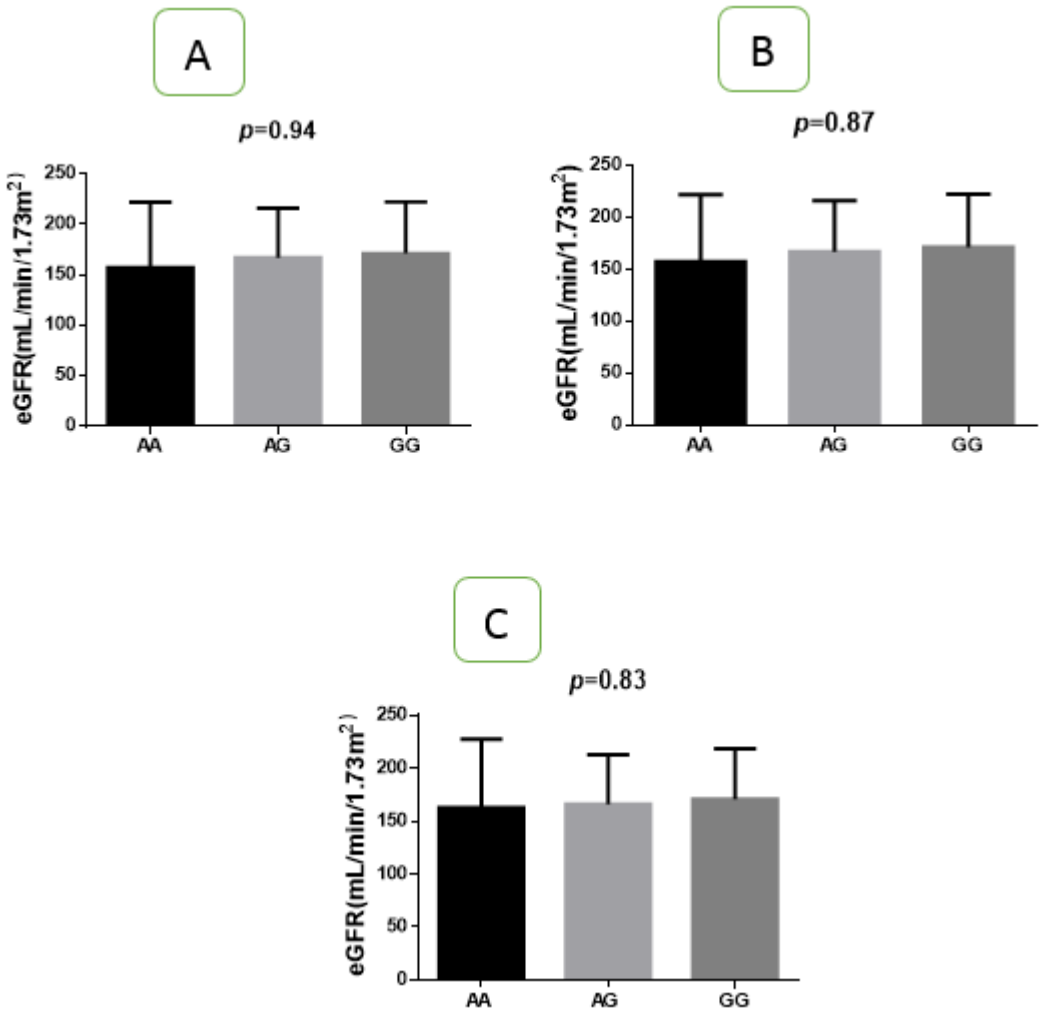

Figure 2: Estimation of the glomerular filtration rate (mL/min/1.73m^2). Calculated by the MDRD method, in patients with sickle cell anemia according to the BMPR1 the X axis represents the BMPR1 genotype and the Y axis to eGFR (Statistical test used, n=75). The values are represented by means ± standard deviation: (A) *p*=0.94 (B) *p*=0.87 and(C) *p*=0.83

9. Appendix 2

Ethical approval

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ANÁLISE DOS POLIMORFISMOS NOS GENES KLOTHO, TNF-alfa, TGF-beta E BMP6 COM O DESENVOLVIMENTO DE ÚLCERAS MALEOLARES EM PACIENTES PORTADORES DE ANEMIA FALCIFORME

Pesquisador: Marcos André Cavalcanti Bezerra

Área Temática:

Versão: 3

CAAE: 05094213.6.0000.5208

Instituição Proponente: Universidade Federal de Pernambuco - UFPE

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.551.708

Apresentação do Projeto:

Trata-se de Emenda para o acréscimo de alguns itens nos objetivos específicos.

A anemia falciforme é a doença hereditária monogênica mais comum do Brasil, ocorrendo, predominantemente entre afro-descendentes. A causa da doença é uma mutação de ponto no gene da globina beta da hemoglobina (Hb), originando uma Hb anormal, a Hb S (HbS), ao invés da Hb normal denominada Hb A (HbA).Considerando a anemia falciforme como uma doença inflamatória crônica. Os polimorfismo nos genes Klotho, TNF-alfa, TGF-ß e no gene BMP6 ,

estão envolvidos com a exacerbação do quadro inflamatório e do estresse oxidativo no endotélio vascular decorrente de processos hemolíticos. A expressão aumentada das citocinas, a diminuição da biodisponibilidade do óxido nítrico, descontrole da proliferação celular e do sistema imune, podem contribuir com a gravidade do curso clínico das úlceras maleolares ou predispor os pacientes à desenvolvê-las. A identificação destes polimorfismos pode ajudar a identificar pacientes com riscos de complicações

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graves,
antes que estas possam ocorrer. Pacientes: a amostra será constituída de 100 pacientes casos e 180 pacientes controles

Objetivo da Pesquisa:

Objetivo Geral

Investigar se existe associação de polimorfismos genéticos com o desenvolvimento de úlceras de membros inferiores, em portadores de anemia falciforme, acompanhados no serviço de Hematologia da Fundação HEMOPE, no estado de Pernambuco.

Objetivos Específicos

• Determinar a frequência dos genótipos resultantes dos polimorfismos nos genes: Klotho (rs685417 e rs516306), TNF- (-308 GA), TGF- (rs1019856) e (rs2038931) e BMP6 (rs270393), na população de pacientes falciformes que desenvolveram ou não úlceras maleolares;
• Investigar se existe associação dos polimorfismos nos genes Klotho (rs685417 e rs516306), TNF- (-308 GA), TGF- (rs1019856) e (rs2038931) e BMP6 (rs270393), com o desenvolvimento de úlceras maleolares em pacientes portadores de anemia falciforme.
• Investigar os polimorfismos no gene da eNOS em pacientes com AF e indivíduos saudáveis.
• Investigar e avaliar se os polimorfismos no gene PADI4 podem estar associados com o risco de desenvolvimento de úlcera de perna em pacientes com AF.
• Determinar a taxa de expressão gênica (em células polimorfonucleares) do PADI4, ELANE e MPO em pacientes com AF e indivíduos saúdaveis.
• Investigar e analisar a influência dos polimorfismos dos genes HMOX1, BMPR1 e APOL1 sobre a taxa de filtração glomerular estimada (eGFR) em pacientes com anemia falciforme (AF) e comparar suas prevalências com as obtidas da população de indivíduos controle saudáveis (HbAA).

Avaliação dos Riscos e Benefícios:

Riscos:

Não haverá riscos graves para os pacientes selecionados. A única intervenção será a de coleta de sangue venoso, devidamente autorizada e realizada por profissional da área. As consequências de uma coleta de sangue venoso são: dor leve e pequenos hematomas.

Benefícios:
Implantação de técnica de pesquisa em biologia molecular que pode ser usada posteriormente na investigação rotineira de pessoas com doenças falciformes, com potencial de desenvolver úlceras de membros inferiores. Os resultados da pesquisa serão divulgadas para o corpo médico do HEMOPE e posteriormente publicada em artigo científico. O(s) polimorfismo(s) que apresentar(em) associação com o desenvolvimento de úlceras
maleolares, serão registrados nos prontuários dos pacientes, e o médico poderá guiar melhor o tratamento destes pacientes.

Comentários e Considerações sobre a Pesquisa:
Trata-se de projeto de interesse clínico no estudo da anemia falciforme.Implantação de técnica de pesquisa em biologia molecular que pode ser usada posteriormente na investigação rotineira de pessoas com doenças falciformes, com potencial de desenvolver úlceras de membros inferiores.

Considerações sobre os Termos de apresentação obrigatória:
O projeto apresenta-se bem fundamentado, com toda a documentação exigida.

Recomendações:
Nenhuma.

Conclusões ou Pendências e Lista de Inadequações:
Aprovado.

Considerações Finais a critério do CEP:
A emenda foi avaliada e APROVADA pelo colegiado do CEP.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1077587_E1.pdf	18/03/2018 19:25:21		Aceito

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

Continuação do Parecer: 2.551.708

Outros	Justificativa_da_Emenda.docx	18/03/2018 19:22:08	Marcos André Cavalcanti Bezerra	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_HEMOPE_CEP_CCS_UFPE_Luana_Laranjeira_modificacoes_amarelo.doc	15/03/2018 15:56:46	Marcos André Cavalcanti Bezerra	Aceito
Outros	Carta resposta às recomendações do CEP.docx	02/01/2014 17:19:25		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE maiores de 18 anos.docx	02/01/2014 16:59:13		Aceito
Outros	Carta de Anuência_HEMOPE.jpg	06/11/2013 22:03:55		Aceito
Outros	Autorização de uso de dados_HEMOPE.jpg	06/11/2013 22:03:11		Aceito
Outros	termo de Confidencialidade_Luana.pdf	31/10/2013 12:34:25		Aceito
Folha de Rosto	Folha de rosto assinada_luana laranjeira.pdf	21/10/2013 20:53:21		Aceito
Outros	Carta de Anuência CCB_luana laranjeira.pdf	21/10/2013 20:50:27		Aceito
Outros	Curriculum Lattes pesquisadores_Luana.docx	10/09/2013 21:50:17		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RECIFE, 20 de Março de 2018

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do Centro de Ciências da Saúde
Bairro: Cidade Universitária **CEP:** 50.740-600
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MINISTÉRIO DA SAÚDE - Conselho Nacional de Saúde - Comissão Nacional de Ética em Pesquisa – CONEP
PROJETO DE PESQUISA ENVOLVENDO SERES HUMANOS

Projeto de Pesquisa:
Investigação das Características Moleculares Associadas à Úlcera de Membros Inferiores em Pacientes com Anemia Falciforme

Informações Preliminares

Responsável Principal

CPF/Documento: 987.061.525-20	Nome: Marcos André Cavalcanti Bezerra
Telefone: 8198008105	E-mail: macbezerra.ufpe@gmail.com

Instituição Proponente

CNPJ: 24.134.488/0001-08	Nome da Instituição: Universidade Federal de Pernambuco - UFPE

Essa submissão de emenda é exclusiva do seu Centro Coordenador?

A emenda não é exclusiva de seu Centro Coordenador, então quando a emenda for aprovada, esta SERÁ replicada nos Centros Participantes vinculados e nos Comitês de Ética das Instituições Coparticipantes.

É um estudo internacional? Não

Equipe de Pesquisa

CPF/Documento	Nome
084.049.544-70	Luana Laranjeira Prado
23867143811	OKEKE CHINEDU

Área de Estudo

Grandes Áreas do Conhecimento (CNPq)

- Grande Área 2. Ciências Biológicas
- Grande Área 4. Ciências da Saúde

Propósito Principal do Estudo (OMS)

- Clínico

Título Público da Pesquisa: ANÁLISE DOS POLIMORFISMOS NOS GENES KLOTHO, TNF-alfa, TGF-beta E BMP6 COM O DESENVOLVIMENTO DE ÚLCERAS MALEOLARES EM PACIENTES PORTADORES DE ANEMIA

Contato Público

CPF/Documento	Nome	Telefone	E-mail
987.061.525-20	Marcos André Cavalcanti Bezerra	8198008105	macbezerra.ufpe@gmail.com

Contato Científico: Marcos André Cavalcanti Bezerra